The Non-Receptor-Associated Tyrosine Kinase Syk is a Regulator of Metastatic Behavior in Human Melanoma Cells

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Melanoma is one of the most aggressive neoplastic transformations and characterized by a high metastatic potential. The current study was performed to assess the impact of ''spleen tyrosine kinase'' (Syk), a non-receptor-associated tyrosine kinase, on growth and metastatic behavior of melanoma cells *in vitro* and in a severe combined immunodeficient (SCID)-mouse/human-melanoma xenotransplantation model *in vivo*. Syk was expressed in melanocytes but was found to be downregulated in melanoma cells. Vector-driven expression of Syk in two different melanoma cell lines did not influence growth speed, but significantly reduced the invasive growth potential of both cell lines in a Matrigel assay *in vitro*. In a SCID-mouse/human melanoma xenotransplantation model, Syk expressing Mel-Juso cells exhibited delayed and reduced tumor growth. After intravenous as well as subcutaneous injection of tumor cells, Syk-transfected cells formed significantly fewer metastatic tumor lesions than control cells. The presented data define Syk as a novel regulator of metastatic behavior of melanoma cells.

Key words: experimental melanoma/neoplasm metastasis/SCID mouse
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"Spleen tyrosine kinase" (Syk) is a member of the group of non-receptor type protein-tyrosine kinases that contains as a specified feature two Src Homology 2 domains located on the amino-terminal end of the protein. Syk is widely expressed in hematopoietic cells, but more recently Syk expression has been noted in multiple non-hematopoietic tissues including the liver (Tsuchida *et al*, 2000), nasal epithelium (Yamada *et al*, 2001), and breast epithelium (Coopman *et al*, 2000). The overwhelming part of functional data however still comes from hematopoietic cells where Syk exerts essential functions in immunoreceptor signalling, natural killer cell-mediated cytotoxicity or maturation of lymphocytes (Cheng *et al*, 1995; Turner *et al*, 1995; Lin *et al*, 1996; Brumbaugh *et al*, 1997).

In breast cancer, Syk expression is absent in invasively growing parts of the tumor. Reconstitution of Syk into Syk-negative breast cancer cells highly reduces their invasive potential. In a mouse model Syk-expressing breast cancer cells formed less metastatic lesions after intravenous injection and exhibited reduced tumor growth after subcutaneous injection (Coopman *et al*, 2000). So far two different mechanisms controlling Syk expression have been described, either hypermethylation of its promoter (Yuan *et al*, 2001) or alternative splicing (Wang *et al*, 2003). The latter results in a splice variant, Syk B, devoid of a nuclear localization signal and inefficient in blocking the metastatic phenotype of breast cancer cells. Alternatively to the nuclear translocation of Syk, a cytoplasmic Syk-regulated pathway has been described to regulate the invasive potential of breast cancer cells. Lack of Syk expression was associated with increased expression of the urokinase type plasminogen activator (uPA) and PI-3 kinase mediated IκBα-phosphorylation after reexpression of Syk blocked uPA expression (Mahabeleshwar and Kundu, 2003). A correlation between these two mechanisms, a nuclear and a cytoplasmic mechanism, respectively, is still missing.

In patients suffering from breast cancer Syk expression is associated with an improved prognosis (Toyama *et al*, 2003). A similar correlation was found in gastric cancer patients, where hypermethylation of the Syk promoter was mainly observed in patients with lymph node metastases (Wang *et al*, 2004).

Melanoma is not only the most malignant tumor of the skin but also one of the most aggressive neoplastic transformations in humans. Although an increasing number of lesions are detected in early stages of the disease and early excision of the tumor is performed (Koh, 1991) there are still a high proportion of patients diagnosed with metastatic melanoma. The 5 y survival rates for patients with clinically detectable lymph node metastases range from 54% to 24% depending on the number of involved lymph nodes and ulceration of the primary tumor (Balch *et al*, 2004). Once dissemination of melanoma beyond the locoregional lymph nodes has taken place the disease is largely incurable. For this reason, increased understanding of mechanisms regulating the metastatic behavior of melanoma is highly important.

The current study was undertaken to assess the expression of Syk in melanoma cells and melanocytes and to test the impact of Syk-expression on the growth and metastatic

Abbreviations: SCID, severe combined immunodeficient; Syk, spleen tyrosine kinase; uPA, urokinase type plasminogen activator

behavior of melanoma cells *in vitro* and in a severe combined immunodeficient (SCID)-mouse/human-melanoma xenotransplantation model *in vivo*.

Results

A 72 kDa band correlating to Syk was readily detected by immunoblotting in three different batches of human melanocytes and in a batch of Epstein–Barr virus transformed B lymphocytes that was used as a positive control. In melanoma cell lines Syk was only detectable at a very low level in SKMel-28 cells, whereas the other melanoma cells tested (607B, 518A2, Mel-Juso, A375, MH, BO, FB, SB) were negative for Syk (Fig 1).

518A2 cells, grown from a melanoma metastasis, Mel-Juso cells, derived from a primary tumor and SKMel-28 cells were stably transfected with either an expression vector (pcDNA3.1-Zeo(+)) containing Syk cDNA (518-syk, Juso-syk, SK28-syk) or with the vector alone (518-zeo, Juso-zeo, SK28-zeo). The selected clones exhibited similar expression levels of Syk and six clones from each group were pooled to avoid subcloning artifacts (Fig 2).

To observe if the expression of Syk had any effect on the growth speed of the transfected cells, an MTS growth assay in 96-well plates was performed. Only a very small difference was observed between 518-zeo and 518-syk cells (Fig 3*B*) and no difference in the amount of cell growth was seen between zeo and syk cells derived from Mel-Juso and SKMel28 (Fig 3*D* and *F*). In contrast a clear difference was observed in a Matrigel invasion chamber assay. Transmigration of Mel-Juso cells upon addition of 2% serum to the lower chamber of the assay system was reduced by $\sim$ 90% in Syk expressing cells (Fig 3*C*, Juso-syk + FCS) as compared with the empty vector control cells (Juso-zeo + FCS). Whereas Juso-zeo and Juso-syk cells had only a very small amount of transmigrating cells without serum addition to the lower chamber (Fig 3*C*, Juso zeo and Juso syk–FCS), 518A2 derived transfectants exhibited spontaneous transmigration even in the absence of a chemotactic gradient (Fig 3*A*, 518-zeo and 518-syk–FCS). Addition of serum doubled the number of transmigrating cells (518-zeo and 518-syk + FCS). Under both conditions, the number of transmigrating cells was reduced by about 50% upon expression of Syk. A similar reduction in the number of transmigrating cells between vector control and Syk expressing cells was observed for SKMel-28-derived cells in the presence of FCS (Fig 3*E*).

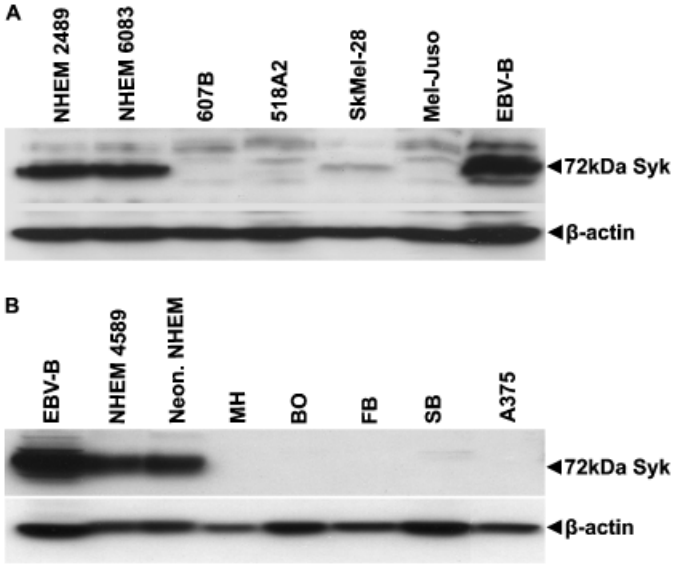

Figure 1
Syk is expressed in melanocytes but not in melanoma cells. (*A*, *B*) Expression of spleen tyrosine kinase (Syk) in four different populations of normal human melanocytes (normal human epidermal melanocytes (NHEM) 2489, 6083, 4589 and neonNHEM) and nine human melanoma cell lines (607B, 518A2, SKMEL-28, Mel-Juso, MH, BO, FB, SB and A375). Epstein–Barr virus transformed B cells (EBV-B) were used as positive control. A polyclonal rabbit anti-Syk antiserum was used for the immunoblot.

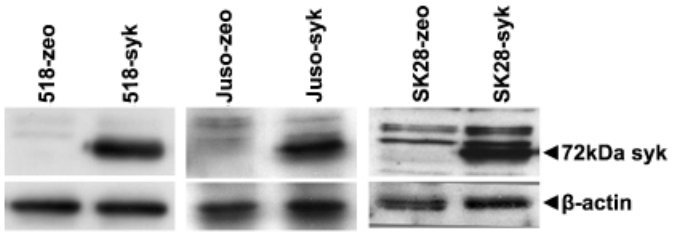

Figure 2
Syk expression in vector-control- and Syk-transfected melanoma cells. Expression of spleen tyrosine kinase (Syk) in 518A2, Mel-Juso and SKMel28 cells stably transfected with either an empty vector (518-zeo, Juso-zeo, SK28-zeo) or the same vector containing the Syk sequence (518-syk, Juso-syk, SK28-syk). A polyclonal rabbit anti-Syk antiserum was used for the immunoblot.

In breast cancer cells, Syk negatively regulates the expression of the uPA by blocking activation of nuclear factor κB (NfκB). No change in the amount of phosphorylated IκBα (Fig 4*C*, P-IκBα) or the subcellular location of p65 (RelA, data not shown) between wild–type (wt), vector control or Syk-transfected cells was observed. We performed a real-time-PCR to assess the amount of uPA–mRNA in Syk-, vector-transfected and wt 518A2, Mel-Juso and SKMel28 melanoma cells. uPA levels were significantly higher in 518A2 cells. Mel-Juso and SKMel28 cells expressed less than 0.02%, 02% of uPA as compared with 518A2 (Fig 4*B*, bars 518-wt, Juso-wt, SK28-wt), and uPA was not detectable in melanocytes (bar normal human epidermal melanocytes (NHEM)). A significant difference was seen between 518-zeo and 518-syk cells ($p = 0.037$) whereas the difference between 518A2 wt cells (518-wt) and 518-syk did not reach significance ($p = 0.109$). Whereas Mel-Juso cells expressed uPA only at a very low level, the difference between Juso-zeo and Juso-syk was significant ($p = 0.05$) and the difference between Juso-wt and Juso-syk was not significant ($p = 0.618$). No significant difference in uPA expression was seen between wt and s*yk*- or vector-transfected cells from the SKMel28 cell line (SK28-zeo *vs* SK28-syk $p = 0.513$; SK28-wt *vs* SK28-syk $p = 0.774$). A real-time PCR for Syk performed from the same samples confirmed the results observed in the immunoblots, namely Syk expression in melanocytes but no expression of Syk in the respective melanoma wt cells (Fig 4*A*, bars 518-wt, Juso-wt, SK28-wt, NHEM).

To check if Syk expression has an influence on the activation of the p42/44 MAP-kinase pathway, an immunoblot specific for the phosphorylated isoform of p42/44 MAP-

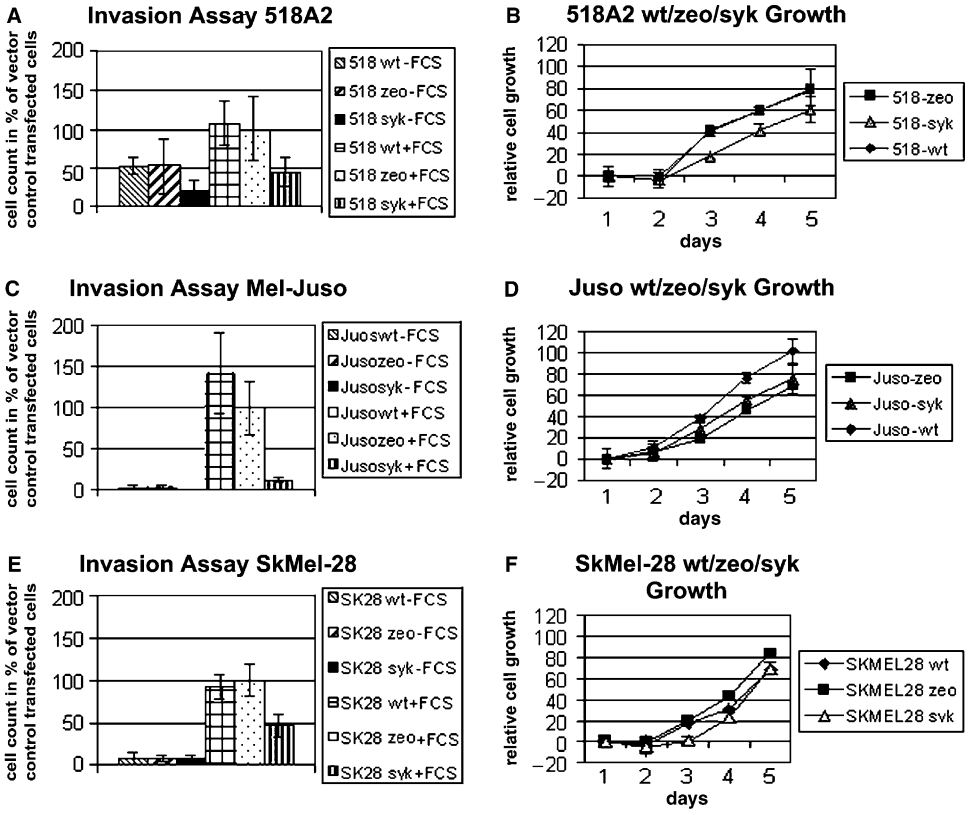

Figure 3
Syk expression reduces the invasive potential of melanoma cells *in vitro.* (*B*, *D*, *F*) MTS assay with spleen tyrosine kinase (Syk) expressing (518-syk, Juso-syk, SK28-syk) and vector control (518-zeo, Juso-zeo, SK28-zeo) cells. Numbers are presented as % of the cell number of the first day after plating the cells. The assays were performed in groups of six; the bars represent SD. (*C*, *D*) Transmigration of melanoma cells in a Matrigel assay with either plain medium (groups − FCS) or medium containing 2% serum (groups + FCS) in the lower chamber of the assay system. Bars represent the number of cells that migrated through the Matrigel layer onto the lower side of the perforated upper chamber. Vector control cells in the + FCS group were set as 100%. Columns present the mean numbers of three independent experiments, and bars represent SD.

kinase was performed. No difference in MAP-kinase activity between controls and Syk transfectants was observed (Fig 4*C*, P-p42/44MAPk and p42/44MAPk).

We furthermore tested the effects of Syk expression on tumorigenity and metastatic potential of human melanoma cells in a SCID-mouse/human-melanoma xenotransplantation model. Vector control and Syk-expressing cells derived from Mel-Juso and 518A2 cells were either injected subcutaneously (sc-groups) into the right flank or intravenously into the animal's tail vein (iv-groups). The primary aim in the sc-groups was to observe if expression of Syk would change the incidence and latency of tumor development. Mice that had received Juso-zeo cells formed palpable tumors 12 d after injection. In contrast, tumor formation by Juso-syk cells was first observed after 21 d (Fig 5*A*). Using 518-zeo and 518-syk cells the time point of tumor take was similar, as the first palpable tumors were observed in both groups 12–14 d after injection. The 518A2 derived transfectants formed larger tumors as compared with the Mel-Juso-derived cell lines, but both groups demonstrated significant differences in tumor size between vector control and Syk-transfected cells (Fig 5*B*), indicating that Syk-derived cells have a reduced ability of tumor formation and tumor growth in an *in vivo* system.

To assess the metastatic potential of the transfected cell lines, 0.5×10^6 cells were injected via the tail vein into SCID mice. The experiment was ended after 40 d when mice in the 518-zeo group developed distress. The lungs, heart, kidneys, spleen, liver, and inguinal lymph nodes were paraffin-embedded and the mean number of metastases was calculated from five representative HE-stained sections. All mice injected with 518-zeo cells and five of six mice injected with 518-syk cells exhibited metastatic tumor spread. The mean number of metastases was 107 ± 30 (mean $\pm$ SD) for 518-zeo *versus* 55 ± 29 for 518-syk ($n = 6$; $p = 0.029$) (Fig 6*A*, *lanes* zeo iv, syk iv). Juso-zeo cells formed metastases in ten of 12 mice with up to 70 lesions in a single animal. In contrast Juso-syk cells formed no more than three metastases per mouse in eight of 12 animals (Fig 6*B*, *lanes* zeo iv, syk iv). The mean number of metastases was 20 ± 22 (mean $\pm$ SD) for Juso-zeo *versus* 1 ± 1 for Juso-syk ($n = 12$; $p = 0.009$).

Internal organs from mice in the groups with subcutaneous tumors were also assessed for metastatic tumor spread. In mice with tumors from 518-zeo and 518-syk cells, a high number of micrometastases (consisting of three to a maximum of 10 cell layers) was observed in the liver. To calculate the number of metastases in the liver of these animals, 10 fields of view with a $\times 100$ magnification were counted on the HE-stained sections and the mean number of micrometastases per field of view per mouse was depicted separately from metastases in other organs (Fig 6*A* *lanes* "sc liver" and "sc non-liver"). The mean number of liver metastases was 84 ± 47 for 518-zeo *versus* 11 ± 3 for 518-syk ($n = 6$; $p = 0.005$). The mean number of metastases in all other organs was 11 ± 4 for 518-zeo *versus* 3 ± 2 ($n = 6$; $p = 0.002$) for mice bearing 518-syk tumors (mean $\pm$ SD).

Reflecting the difference between a cell line derived from a primary tumor and a metastasis, the groups with subcutaneously injected Mel-Juso transfectants formed significantly less metastases than 518A2 cells but still demonstrated a difference between vector control and Syk expressing cells. The mean number of metastases was 6 ± 7 for Juso-zeo *versus* 1 ± 1 for Juso-syk (mean $\pm$ SD, $n = 12$, $p = 0.023$). Hence, our data show that the expression of Syk

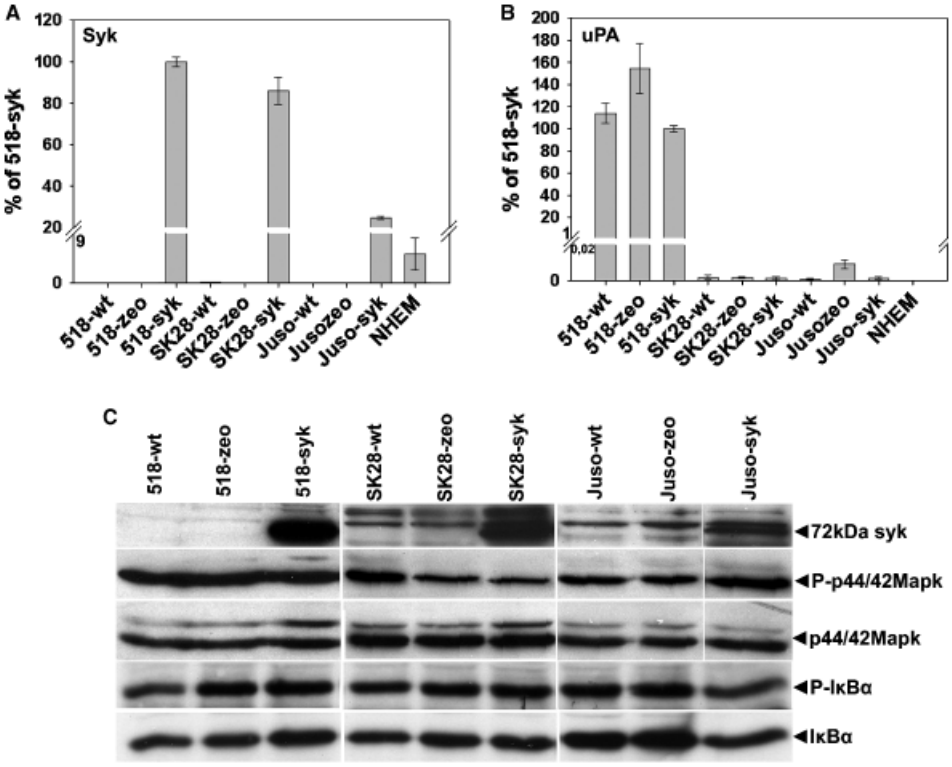

Figure 4
Syk does not regulate uPA expression, 1κBα-phosphorylation or phosphorylation of p44/42 MAP-kinase in melanoma cells. (*A*) Quantification of spleen tyrosine kinase (Syk)- and urokinase type plasminogen activator (uPA)-mRNA in melanocytes (normal human epidermal melanocytes, NHEM) and in wild-type-(-wt), vector control-(-zeo) and Syk-transfected-(-syk) 518A2, SKMel28 and Mel-Juso cell lines by real-time PCR. Results are expressed in percentage relative to 518 syk. (*B*) Western blot with antibodies specific for phosphorylated and unphosphorylated p42/44 MAP-kinase (*lanes* P-p42/44MAPk and p42/44MAPk) as well as phosphorylated and unphosphorylated IκBα (*lanes* P-IκBα and IκBα) in wild-type-(-wt), vector control-(-zeo) and Syk-transfected-(-syk) 518A2, SKMel28 and Mel-Juso cell lines. Syk expression is shown in the upper lane.

not only influences the growth of tumor lesions after subcutaneous and intravenous injection but also controls the formation of spontaneous metastases from an established tumor grown from human melanoma cells *in vivo.*

Discussion

Patients suffering from human melanoma rarely succumb because of local effects of the primary tumor but usually because of a progressive loss of organ function from metastatic tumor growth. Metastatic growth itself is a multistep process, and some of the mechanisms controlling this process have been elucidated.

In our work, we describe the influence of the non-receptor-associated tyrosine-kinase Syk on the metastatic behavior of melanoma cells. The complete loss or substantial downregulation of Syk-expression in all melanoma cells as opposed to its higher expression in melanocytes indicates that Syk is downregulated in the process of melanoma oncogenesis. The difference in metastatic potential observed after vector-derived reconstitution of Syk expression in two of the melanoma cell lines indicates that Syk is a negative regulator of metastatic behavior in melanoma cells. These results were observed only in the "three-dimensional" Matrigel-assay system, but no difference in cell proliferation in a "two-dimensional" culture plate was observed. This correlates well with results observed under similar conditions in breast cancer cells (Coopman *et al*, 2000). Whereas Syk positive tumor cell lines have been described for breast cancer all melanoma cell lines tested were negative for Syk, irrespective of whether they were derived from a primary tumor, a lymph node, subcutaneous or distant metastasis. This implies that loss of Syk expression is a basic mechanism influencing the metastatic behavior of melanoma cells and that further mechanisms add to the final metastatic potential of the cells.

In 518A2 transfectants, transmigration of cells in the absence of a chemotactic gradient was inhibited as well by the expression of Syk. This, together with the lack of an effect on growth speed, leads to the conclusion that the effect of Syk must be targeted to the process of active transmigration and does not impinge on pathways regulating the reaction to chemotactic signals or cell cycle progression. Syk-dependent regulation of uPA, a secreted enzyme enabling degradation of extracellular matrix (Sidenius and Blasi, 2003), via the NfκB pathway was demonstrated in MCF-7 and MDA-MB-23 breast cancer cell lines (Mahabeleshwar and Kundu, 2003). In contrast to breast cancer, none of the melanoma cells expressing Syk after transfection, despite having a clearly reduced metastatic potential, showed any change in the phosphorylation of IκBα. Expression of uPA showed statistically significant differences between vector control and Syk-transfected cells in two of the three cell lines investigated (518A2, Mel-Juso). However several factors argue against a role of uPA expression as the underlying mechanism in melanoma cells: (I) the difference was only significant between vector control and Syk-transfected cells but not between wt and Syk-transfected cells, (II) one cell line (SKMel-28) did not exhibit changes in uPA expression despite clearly changing its invasive potential after Syk transfection, (III) the extent of uPA regulation did not show a consistent correlation with the high differences seen in Syk expression between control and Syk-transfected cells, (IV) the overall level of uPA was very low in SKMel-28 and Mel-Juso cells as compared with 518A2 cells making a major contribution of uPA in these cells questionable, and (V) the different levels of uPA in the wt cells did not correlate to the

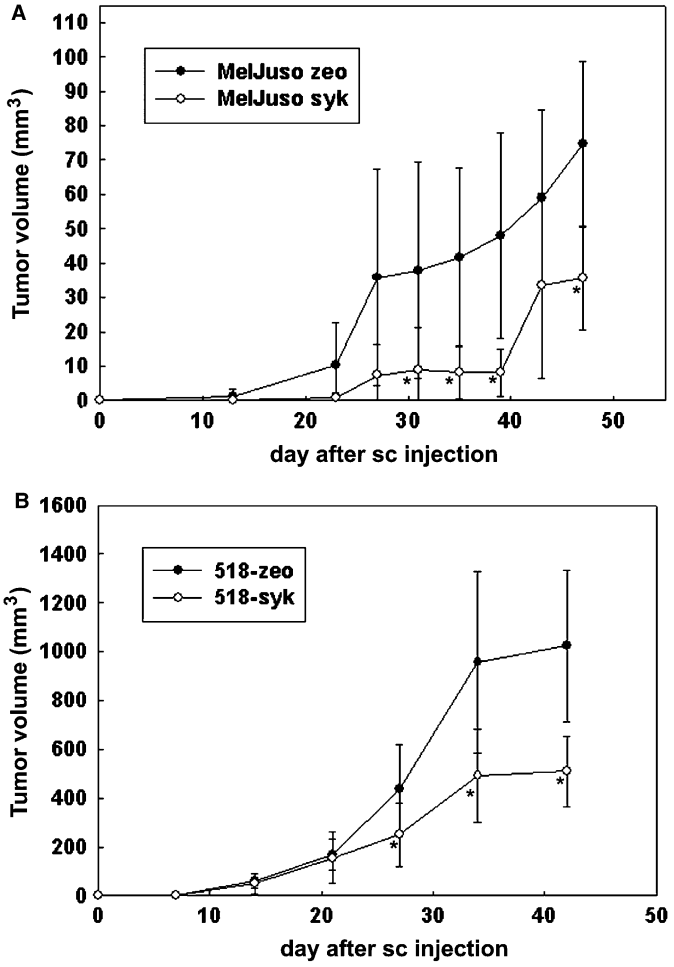

Figure 5
Syk-expression leads to reduced tumor growth after subcutaneals injection of melanoma cells in SCID-mouse xenotransplantation model. (*A*, *B*) Tumor volume of subcutaneous tumors formed after injection of either spleen tyrosine kinase (syk) expressing (Juso- and 518-syk) or vector control (Juso- and 518-zeo) cells into the right flank of severe combined immunodeficient mice. Values represent the mean tumor volume per group ± SD. Values in the Syk groups marked with * were significantly different from the respective vector control cells by paired student's *t*-test.

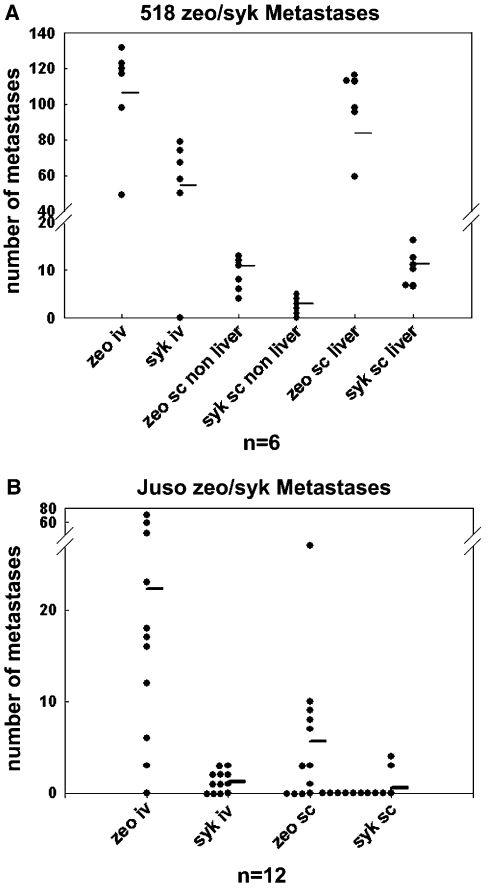

Figure 6
Syk-expression is associated with a reduced number of metastases in a SCID-mouse xenotransplantation model. (*A*, *B*) Number of metastases in severe combined immunodeficient mice after iv injection of either spleen tyrosine kinase (Syk) expressing or vector control melanoma cells (zeo iv, syk iv) as well as in mice bearing subcutaneous tumors from these cells (zeo sc, syk sc). Lines represent the mean number of metastases in each group. Because of a high number of micrometastases in the livers of mice with subcutaneous tumors formed by 518-zeo or 518-syk cells, these were depicted separately as the mean number of lesions from 10 fields of view in a × 100 magnification (zeo sc liver and syk sc liver).

missing differences in Syk expression between these cells. Taken together this indicates that other pathways regulating active transmigration might be regulated by Syk in melanoma. Activation of the p42/44 MAP-kinase, another important pathway that regulates tumor progression, expression of matrix metalloproteases and tumor angiogenesis in melanoma (Cohen *et al*, 2002; Govindarajan *et al*, 2003), was however also not regulated by Syk expression in melanoma cells.

The *in vitro* results are substantiated by the results from the *in vivo* experiments. Juso-syk cells exhibited a delayed tumor take and reduced tumor size after subcutaneous injection into SCID mice. In contrast, the time point of tumor take was similar between 518-zeo and 518-syk cells, which might reflect their higher "basal" invasive potential that was observed in the *in vitro* assays. Despite this, the tumor volume was also significantly smaller in the Syk expressing cells. A model system that could explain both observations (the delayed tumor take as well as the reduced tumor growth) is that secretion and activation of matrix degrading enzymes and subsequent "remodelling" of the tumor environment is an essential prerequisite for local tumor growth (Hendrix *et al*, 2003; Hendrix *et al*, 2003). It would therefore not only influence the time point of tumor establishment but also exert an ongoing influence on tumor growth.

In both cell systems, the number of metastases was significantly reduced in Syk-expressing cells after intravenous injection of tumor cells into the tail vein of SCID mice. The difference in the mean number of metastases was similar to the difference observed in invasive potential *in vitro*. Notably, a difference in the number of metastases was also observed after spontaneous tumor spread in mice bearing subcutaneous tumors thereby further supporting the role of Syk as a regulator of melanoma metastases formation.

This is a report that the non-receptor-associated tyrosine kinase p72 Syk is a regulator of the metastatic behavior of melanoma cells. Additionally, the presented data suggest that loss of Syk expression that up to now was described in breast and gastric cancer, might be a more general phenomenon in malignant disease. Ongoing work will have to

focus on identifying further cellular pathways that are associated with Syk influence on metastases.

Materials and Methods

Tumor cell lines 518A2, 607B (courtesy of Dr Peter Schrier, Leiden, the Netherlands), SKMEL-28, A375 (ATCC), Mel-Juso (German Collection of Microorganisms and Cell Culture, DSMZ, Braunschweig, Germany) FB, MH, SB, BO (isolated from a nodular primary tumor (MH), subcutaneous metastasis (SB), ovaric metastasis (FB) and lymph node metastasis (BO) and characterized for their expression of S100, HMB45 and Tyrosinase) melanoma cells were maintained in Dulbecco's mofified Eagle's medium (DMEM) (Invitrogen, Carlsbad, California) supplemented with 8% heat-inactivated FCS. NHEM were obtained as cryopreserved cells from Clonetics/Cambrex (East Rutherford, New Jersey). NHEM were maintained in MBM-2 medium supplemented with the MGM-3 bullet kit (all Clonetics) and were used between the third and sixth doubling after thawing. 518A2, SKMel-28 and Mel-Juso cells were transfected with the human Syk cDNA containing mammalian expression vector pcDNA3.1-Zeo(+) that was kindly provided by Professor Susette C Mueller (Georgetown University Medical Center, Washington). Cells were transfected using a cationic lipid (Lipofectin, Invitrogen) according to the manufacturers instructions. Four hundred microgram per mL Zeocin (Invitrogen) was used for selection of transfected cells that were tested for Syk expression by western blotting. To avoid subcloning artifacts, six individual clones were pooled.

Western blot Samples were prepared using a buffer containing 150 mM NaCl, 1% NP 40%, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris, 2 mM PMSF, 2 mM Na_3O_4V and complete protease inhibitors (Roche Diagnostics, Basel, Switzerland). Western blots were performed using 30 μg of protein. Prior to application protein lysates were cooked at 95°C for 10 min in equal volumes of a 2 × loading buffer (30 mM Tris, 12.5% glycerol, 1% SDS, 12.2 mM mercaptoethanol, 0.5% bromophenol blue). Proteins were blotted onto a nitrocellulose membrane. Ponceau-red stain and an antibody directed against β-actin were used as loading controls. Antibodies used were: anti-Syk rabbit polyclonal (N19, Santa Cruz Biotechnology, Santa Cruz, California), anti–[phospho p42/44 MAP-kinase mouse monoclonal, anti-p42/44 MAP-kinase rabbit polyclonal (Cell Signaling, Beverley, Massachusetts) anti-phospho-IκBα rabbit polyclonal, anti-IκBα rabbit polyclonal and anti-actin rabbit polyclonal (Sigma, St Louis, Missouri). To assure that none of the differences observed between melanocytes and melanoma cells was because of different culture conditions melanoma cells were grown in melanocyte medium as well as in the routinely used DMEM medium 48 h prior to harvesting. No differences were observed (data not shown).

Matrigel invasion chamber assay The assay (BD Biosciences, Bedford, Massachusetts) consists of a two-well chamber system, where the upper chamber has a perforated bottom with a pore-size of 8 μm, large enough to allow cells to migrate through these pores. Overlying the perforated bottom of the upper well is an artificial basement membrane preparation (originally derived from Engelbreth–Holm–Swarm mouse sarcomas). 25×10^3 cells were plated into the upper chamber in serum-free medium. The upper chamber was placed inside the lower chamber which contained either serum-free medium or medium with 2% FCS to create a chemotactic gradient. After 24 h, the upper chamber was removed, Matrigel and remaining cells were cleared by swiping with a cotton bud, and the transmigrated cells on the lower side of the upper chamber were fixed in 70% ethanol and stained using 0.2% crystal blue. Cells in ten × 40 fields were counted from every well and expressed as mean ± SD.

Growth assay Two thousand five hundred cells in a final volume of 100 μL were plated in 96-well plates and cell proliferation was measured photometrically using the Cell titer 96 assay (Promega, Madison, Wisconsin). This assay is composed of a tetrazolium compound (MTS, Owens reagent) and an electron-coupling reagent (phenazine methosulfate, PMS). MTS is bioreduced by cells into a formazan that is soluble in tissue culture medium and absorbance of the formazan can be measured at 490 nm.

Mouse experiments All procedures involving experimental animals were performed according to protocols approved by the committee on animal welfare of the Medical University of Vienna. Groups of either six or twelve 7–8 wk old female, pathogen-free C.B 17-SCID mice (Harlan-Winckelmann, Germany) were either injected with 10×10^6 Syk-transfected or vector control melanoma cells into the subcutaneous fat pad of the right flank, or 0.5×10^6 cells were injected intravenously via the tail vein. Animals were sacrificed after a maximum of 50 d or when animals in one group developed serious distress. Internal organs were explanted, fixed in 7.5% formaldehyde, paraffin-embedded and five representative HE-stained sections 300–500 μM apart were observed for metastatic lesions. Tumor volume in the subcutaneous tumor groups was calculated by the formula [(largest diameter × (smallest diameter)^2) × 1/6π] and depicted as mean tumor volume per group ± SD. Results of Syk transfectants and vector controls were compared for significance using the *t*-test. A $p \leqslant 0.05$ was considered as statistically significant.

Real-time RT-PCR Primers for SYK and uPA as well as for human glyceraldehyde 3-phosphate dehydrogenase as endogenous control were ordered from Applied Biosystems (Foster City, California) (SYK: Hs00374292_m1; uPA: Hs00170182_m1). ABI PRISM 7700 Sequence Detection System was used for amplification and detection. Reactions were performed in 20 μL and standard thermal cycler conditions were chosen with 2 min 50°C; 10 min 95°C; 45 cycles 15 s 95°C, 1 min 60°C. Three independent experiments were conducted. To check whether the primers are applicable to multiplex PCR (target and endogenous control amplified in the same tube) for both SYK and uPA separate and multiplex reactions were processed simultaneously. No non-specific products were detected in multiplex PCR permitting its use in subsequent analyses. Reverse transcription negative controls (RT minus, isolated RNA without reverse transcriptase) were also subjected to PCR to rule out target amplification from genomic DNA contamination. For relative quantitation of gene expression, comparative C_T method was selected (as described in ABI User Bulletin #2, p11ff. http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf).

Statistical analysis was performed by paired *t*-test.

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References

Balch CM, Soong SJ, Atkins MB, *et al*: An evidence-based staging system for cutaneous melanoma. CA Cancer J Clin 54:131–149, 2004

Brumbaugh KM, Binstadt BA, Billadeau DD, Schoon RA, Dick CJ, Ten RM, Leibson PJ: Functional role for Syk tyrosine kinase in natural killer cell-mediated natural cytotoxicity. J Exp Med 186:1965–1974, 1997

Cheng AM, Rowley B, Pao W, Hayday A, Bolen JB, Pawson T: Syk tyrosine kinase required for mouse viability and B-cell development. Nature 378:303–306, 1995

Cohen C, Zavala-Pompa A, Sequeira JH, *et al*: Mitogen-activated protein kinase activation is an early event in melanoma progression. Clin Cancer Res 8:3728–3733, 2002

Coopman PJ, Do MT, Barth M, *et al*: The Syk tyrosine kinase suppresses malignant growth of human breast cancer cells. Nature 406:742–747, 2000

Govindarajan B, Bai X, Cohen C, *et al*: Malignant transformation of melanocytes to melanoma by constitutive activation of mitogen-activated protein kinase kinase (MAPKK) signaling. J Biol Chem 278:9790–9795, 2003

Hendrix MJ, Settor EA, Kirshmann DA, Quaranta V, Settor RE: Remodeling of the microenvironment by agressive melanoma tumor cells. Ann NY Acad Sci 995:151–161, 2003

Koh HK: Cutaneous melanoma. N Engl J Med 325:171–182, 1991

Lin S, Cicala C, Scharenberg AM, Kinet JP: The Fc(epsilon)RI beta subunit functions as an amplifier of Fc(epsilon)RIgamma-mediated cell activation signals. Cell 85:985–995, 1996

Mahabeleshwar GH, Kundu GC: Syk, a protein-tyrosine kinase, suppresses the cell motility and nuclear factor kappa B-mediated secretion of urokinase type plasminogen activator by inhibiting the phosphatidylinositol 3′-kinase activity in breast cancer cells. J Biol Chem 278:6209–6221, 2003

Sidenius N, Blasi F: The urokinase plasminogen activator system in cancer: Recent advances and implication for prognosis and therapy. Cancer Metastasis Rev 22:205–222, 2003

Toyama T, Iwase H, Yamashita H, *et al*: Reduced expression of the Syk gene is correlated with poor prognosis in human breast cancer. Cancer Lett 189: 97–102, 2003

Tsuchida S, Yanagi S, Inatome R, *et al*: Purification of a 72-kDa protein-tyrosine kinase from rat liver and its identification as Syk: Involvement of Syk in signaling events of hepatocytes. J Biochem 127:321–327, 2000

Turner M, Mee PJ, Costello PS, *et al*: Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. Nature 378:298–302, 1995

Wang L, Duke L, Zhang PS, *et al*: Alternative splicing disrupts a nuclear localization signal in spleen tyrosine kinase that is required for invasion suppression in breast cancer. Cancer Res 63:4724–4730, 2003

Wang S, Ding YB, Chen GY, Xia JG, Wu ZY: Hypermethylation of Syk gene in promoter region associated with oncogenesis and metastasis of gastric carcinoma. World J Gastroenterol 10:1815–1818, 2004

Yamada T, Fujieda S, Yanagi S, Yamamura H, Inatome R, Sunaga H, Saito H: Protein-tyrosine kinase Syk expressed in human nasal fibroblasts and its effect on RANTES production. J Immunol 166:538–543, 2001

Yuan Y, Mendez R, Sahin A, Dai JL: Hypermethylation leads to silencing of the SYK gene in human breast cancer. Cancer Res 61:5558–5561, 2001